EFFECTS OF USING ENHANCED BIOFERTILIZER CONTAINING N-FIXER BACTERIA ON PATCHOULI GROWTH

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I declare that this thesis entitled "Effects of Using Enhanced Biofertilizer Containing N-fixer Bacteria on Patchouli Growth" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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Special Dedication to my family

For all your care, support and believe in me.

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ABSTRACT

Producing effective biofertilizer is crucial for environment and future demands while patchouli is important essential oil plants in the world. The objective of this study was to evaluate the effect of enhancing biofertilizer with N-fixer bacteria by applying on patchouli plant. In this study, growth profile of screened N-fixer bacteria is obtained to harvest highest yield of active bacteria. Then, N-fixer strain is added in purchased biofertilizer using peat moss carrier. After 2 weeks, the nitrogen content is determined by Kjeldahl method. The effectiveness of this modified biofertilizer is indicated by average physical changes of plants. From analysis, it was found that nitrogen content of biofertilizer is increased linearly with the increase of N-fixer percentage inoculum. Using biofertilizer can offer yield as good as using chemical fertilizer if complex biofertilizer containing different strain is used. Application of enhanced biofertilizer on patchouli showed improvement of leaves and branch growth up to 8% and 5% respectively compared to original biofertilizer. The suitable range of N-fixer inoculum should be applied to biofertilizer is more than 10%.

ABSTRAK

Penghasilan baja bio yang berkesan adalah amat kritikal untuk alam sekitar dan permintaaan pada masa hadapan manakala patchouli diklasifikasikan sebagai tumbuhan berharuman penting di dunia. Objektif kajian ini adalah untuk menilai kesan penggunaan baja bio yang ditambahbaikkan dengan bakteria pengikat nitrogen dengan cara mengaplikasikannya terhadap pokok patchouli. Dalam kajian ini, profil pertumbuhan bakteria pengikat nitrogen ditentukan untuk memperolehi sebanyak mungkin bakteria yang aktif. Kemudian bakteria tersebut dicampur dengan pembawa lumut-gambut dan dibiarkan selama 2 minggu sebelum kandungan nitrogen dalam baja bio ditentukan dengan kaedah Kjeldahl. Keberkesanan baja bio ini dapat ditunjukkan dengan perubahan fizikal purata tumbuhan. Daripada analisis, didapati bahawa kandungan nitrogen di dalam baja bio meningkat dengan setiap peningkatan peratusan inokulum N-fixer. Penggunaan baja bio dapat memberi kesan sebaik baja kimia jika baja bio kompleks yang memgandungi pelbagai spesies digunakan. Aplikasi baja bio yang ditambahbaikkan terhadap patchouli menunjukkan 8% dan 5% peningkatan berlaku kepada pertumbuhan daun dan cabang berbanding baja bio yang asal. Had inokulum N-fixer yang sesuai diaplikasikan kepada baja bio adalah 10% dan keatas.

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LIST OF SYMBOLS/ABBREVIATIONS

DNS	-	Di-Nitro Salicylic Acid
DI	-	deionized
g	-	gram
g/L	-	gram per liter
K	-	potassium
L	-	liter
mg/L	-	milligram per liter
min	-	minutes
mL	-	mililiter
N	-	nitrogen
N	-	Normality
N-fixer	-	nitrogen fixer bacteria
nm	-	nanometer
OD	-	optical density
P	-	phosphorus
rpm	-	rotation or revolution per minute
v/v	-	volume per volume
v/w	-	volume per weight
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

In this chapter we will discuss about background of study, objective, scope, problem statement and also rationale and significance of the research.

1.1 Background of Study

Fertilizer is widely used to supply essential nutrient for plant to increase yield. In fact, yields of most crop plants are increase linearly with the amount of fertilizer that they absorb (Loomis and Conner, 1992). Due to this fact, agricultural sector are strongly depending on fertilization with mineral nutrients. When crop plants are grown under modern high-production conditions, substantial amounts of nutrients are removed from the soil (Taiz and Zeiger, 2002). Then, to prevent deficiencies, nutrients can be added back to the soil in the form of fertilizers.

There are many types of fertilizer that existed such as inorganic fertilizer, organics fertilizer and biofertilizer. Fertilizers that provide nutrients in inorganic forms are called chemical fertilizers and those that derive from plant or animal residues are considered as organic fertilizers (Taiz and Zeiger, 2002). Biofertilizer are the products containing living cells of different types of microorganisms which have an ability to mobilize nutritionally important elements from non usable to usable form through biological process (NIIR Board, 2004). Among of these fertilizers, chemical fertilizer is the most extensively used in plantation.

Global fertilizer consumption is supported by an exponential growth in biofuels crops and a recovery in fertilizer use in the main consuming regions. Grain consumption is rising, driven by strong demand for food, feed and biofuels production, leading to very tight grain market conditions and a severe contraction of the world stock-to-use ratios well below critical levels (Maene, 2007).

However, extensive used of chemical fertilizers in long term cause declining in productivity and also environmental quality (Rahim, 2002). In the light of these problems, the use of organic fertilizers, biofertilizer and other microbial products is crucial in the current attempt to make the agriculture industry a viable component of a healthy and pleasant ecosystem (Rahim, 2002). Improvement should be done to these fertilizers to replace the usage of chemical fertilizer. Thus, this undergraduate research project is set up to improve the biofertilizer effectiveness to make it have superior potency over the chemical fertilizer.

1.2 Objective

The aim of this research is to modifiy and enhances biofertilizer by applying active nitrogen fixer (N-fixer) bacteria inside it. Thus, the objectives of this research are:

i. To obtain N-fixer growth profile in batch fermentation
ii. To obtain optimum N-fixer percentage inoculums can be applied in modified biofertilizer based on physical changes of plants.
iii. To compare the effect using enhanced biofertilizer containing different percentage of N-fixer inoculums with unmodified biofertilizer, chemical and organics fertilizer

1.3 Scope of Study

The scope of this research are to determine the effect of N-fixer inoculums in modified biofertilizer and compare it effectiveness with others fertilizer. Firstly, N-fixer growth profile is obtained by fermentation to gain active N-fixer. Then, N-fixer is added in biofertilizer with different percentage before applying on patchouli plants. Changes of nitrogen content in biofertilizer will be determined by Kjeldahl method. The effectiveness of enhanced biofertilizer will be tested on patchouli and proved by its physical changes. The techniques that will be employed include glucose assay, OD reading and physical changes observation. The equipment involved is UV-Vis Spectrophotometer, Kjeldahl apparatus and measuring set.

1.4 Problems Statement

The nature and the characteristics of nutrient release of chemical fertilizer, organic fertilizer and biofertilizers are different, and each type of fertilizer has its advantages and disadvantages with regard to crop growth and soil fertility (Chen, 2008). Chemical fertilizers are commonly used in plantation areas or farms because the nutrients are soluble and immediately available to the plants; therefore the effect is usually direct and fast (Chen, 2008). Besides, the price is lower and more competitive than organic fertilizer, which makes it more acceptable and often applied by users (Chen, 2008).

As a comparison, organic fertilizer production often has difficulties to standardize its constituent and the nutrient release rate is too slow to meet crop requirements in a short time, hence some nutrient deficiency may occur (Chen, 2008). Nevertheless, the usage of organics fertilizer does not leaved residues that can cause harm to environment like chemical fertilizer because they release nutrients slowly and contribute to the residual pool of organic N and P in the soil, reducing N leaching loss and P fixation (Chen, 2008). Chemical fertilizer does gives good yield but deteriorate our environment (Rahim, 2002).

The dilemma that fertilizer consumer face is to choose fertilizer that can give higher yield but cause harm to environment or to choose fertilizer that can preserve the environment but give slower effect. As a winding up, biofertilizer can be used to substitute organics and chemical fertilizer. Since it does not cause pollution like chemical fertilizer and give faster effect compare to organics fertilizer, it should be put at the first place. The technology for biofertilizers production is also relatively simple and installation cost is very low compared to chemical fertilizer plants.

However, the application of microbial fertilizers in practice, somehow, not achieved constant effect. The mechanism and interaction among the microbes still are not well understood, especially in real application (Wu *et al.*, 2004). The yields of plant that are using biofertilizer are still lower compare to using chemical fertilizer. Hence some modification needs to be done to enhance biofertilizer itself. This research is conducted to determine the effect of biofertilizer enhanced with N-fixer inoculums on patchouli growth. It is desire to obtain optimum percentage of inoculums to be added in biofertilizer within stated range.

1.5 Rationale and Significance

Fertilizers are global commodities and the price and availability are influenced by lot of factors throughout the world. In second half of 2008, it is predicted that South Asia, Eastern Europe and Central Asia will increase their fertilizer demand by more than 3% in 2008 and 2009 (Heffer and Prud’homme, 2008). Driven by strong demand for bio-fuels crops and a recovery of fertilizer use in the main consuming regions, global fertilizer consumption in 2006 rose 6 metric tonnes nutrient over 2005, to 161.8 metric tones (Maene, 2007).

At the regional level, the bulk of the increase in demand is expected to come from Asia and Latin America. South Asia and East Asia together would account for one third of total growth. In East Asia, regional demand will remain firm 2.9 % increase annually due to usage in China, Indonesia, Malaysia and Vietnam (Maene, 2007). As the major world palm oil producer, Malaysia and Indonesia is likely to have strong demand for fertilizer. Growth in the economies of China and India, in particular, as well as other countries has created a greater worldwide demand for fertilizers, and increased use of corn for ethanol production is increasing fertilizer demand in the United States due to expected increases in corn acres (Virginia State University, 2007).

World production capacity for nitrogen and phosphorus fertilizers is slightly greater than demand while potassium capacity is significantly greater than demand, but the production has been constrained by several factors in recent years including price (Virginia State University, 2007). As the base of the chemical fertilizers is fossil fuel, the prices of chemical fertilizer will goes up due to limited un-renewable resources. As a consequence, many countries are now shifting to use biofertilizer that have longer lasting effect compared to chemical fertilizer. Lot of researches and paperwork is done to fulfill world biofertilizer demand.

Some agreements that have been made by certain countries such as Kyoto Agreement in 1997 also give large opportunities for biofertilizer to be expanded. It is participated by 140 countries which agreed to reduce the usage of chemical fertilizer started by year 2010. Enhancement and large scale production of biofertilizer will helps to replace the usage of chemical fertilizer that can cause harm to environment and replace the usage of organics fertilizer that sluggish. This will definitely added the value of biofertilizer itself in the market and as a result, consumer will definitely choose biofertilizer instead of others fertilizer.

CHAPTER 2

LITERATURE REVIEW

This chapter will elaborate and summarize the literature reading for related major topics. Some explanation for the minor sub-topics is also included to give overall figure of this research.

2.1 Biofertilizer

2.1.1 History and Background

The term biofertilizer or called 'microbial inoculants' can be generally defined as a preparation containing live or latent cells of efficient strains of nitrogen fixing, phosphate solubilizing or cellulytic microorganisms used for application of seed, soil or composting areas with the objective of increasing the numbers of such microorganisms and accelerate certain microbial process to augment the extent of the availability of nutrients in a form which can assimilated by plant (NIIR Board, 2004). In large sense, the term may be used to include all organic resources (manure) for plant growth which are rendered in an available form for plant absorption through microorganisms or plant associations or interactions (NIIR Board, 2004).

The knowledge of applied microbial inoculums is long history which passes from generation to generation of farmers. It started with culture of small scale compost production that has evidently proved the ability of biofertilizer. This is recognize when the cultures accelerate the decomposition of organics residues and agricultural by-products through various processes and gives healthy harvest of crops (Rahim, 2002). In Malaysia, industrial scale microbial inoculants are started in the late 1940's and peaking up in 1970's taking guide by *Bradyrhizobium* inoculation on legumes (Rahim, 2002). Government research institute, the Malaysian Rubber Board (MRB) had been conducting research on *Rhizobium* inoculums for leguminous cover crops in the inter rows of young rubber trees in the large plantations. Besides, Universiti Putra Malaysia (UPM) also has conducted many researches since 1980's on *Mycorrhiza* and initiated the research to evaluate the contribution of nitrogen from *Azospirillum* to oil palm seedlings (Rahim, 2002).

Rahim (2002) reported that *Mycorrhiza* inoculums are the biofertilizer that is increasingly being utilized and accepted in agriculture industry of Malaysia. It was also reported that a decomposer fungus *Trichoderma reesei*, combine with the nitrogen fixing bacterium *Azotobacter* produce compost within shorter time and give high nitrogen count. This can helps to fertilize unproductive sandy soil and spoiled land that result from mining activities because biofertilizer can helps to supply nutrient continuously. Large scale productions of biofertilizer are produced mainly for supplying nutrient, amelioration of toxic effect in soils, root pest and disease control, improved water usage and soil fertility (Rahim, 2002). Since the substrate for inoculate are abundant such as mine sands and agricultural wastes, the production cost is cheaper and environmentally safe.

There are lot of perception is lay on biofertilizer. It is often perceived to be more expensive than the chemical fertilizers due to the lack of skills and technology to produce biofertilizer products from abundant wastes (Rahim, 2002). Besides, the effect on the crops is slow, compared to chemical fertilizers. Special care such as storage or mixing with powders is also needed to handle microbial inocula to make they remain effective for extended use. As biofertilizers contain living organisms, their performance therefore depends on environment surrounding. Hence, outcomes are bound to be inconsistent (Rahim, 2002). Short shelf life, lack of suitable carrier materials, susceptibility to high temperature, problems in transportation and storage are biofertilizer bottlenecks that still need to be solved in order to obtain effective inoculation (Chen, 2008).

2.1.2 Production of Biofertilizer

There are several things need to be considered in biofertilizer making such as microbes' growth profile, types and optimum condition of organism, and formulation of inoculum. The formulation of inocula, method of application and storage of the product are all critical to the success of a biological product (Chen, 2008).

In general, there are 6 major steps in making biofertilizer. These includes choosing active organisms, isolation and selection of target microbes, selection of method and carrier material, selection of propagation method, prototype testing and large scale testing. First of all, active organisms must be decided. For example, we must decide to use whether organic acid bacteria or nitrogen fixer or the combination of some organisms. Then, isolation is made to separate target microbes from their habitation. Usually organism are isolate from plants root or by luring it using decoy such as putting cool rice underground of bamboo plants.

Next, the isolated organisms will be grown on Petri plate, shake flask and then glasshouse to select the best candidates. It is also important to decide form of our biofertilizer product wisely so that the right carrier material can be determined. If it is desired to produce biofertilizer in powder form, then tapioca flour or peat are the right carrier materials. Selection of propagation method is mainly to find out the optimum condition of organism. This can be achieved by obtaining growth profile at different parameter and conditions. After that, prototype (usually in different forms) is made and tested. Lastly, biofertilizer is testing on large scale at different environment to analyze its effectiveness and limitability at different surrounding.

2.1.2.1 Common Organism Used in Biofertilizer

Organisms that are commonly used as biofertilizers component are nitrogen fixers (N-fixer), potassium solubilizer (K-solubilizer) and phosphorus solubilizer (P-solubilizer), or with the combination of molds or fungi. Most of the bacteria included in biofertilizer have close relationship with plant roots (FNCA Biofertilizer Project Group, 2006). *Rhizobium* has symbiotic interaction with legume roots, and *Rhizo*-bacteria inhabit on root surface or in rhizosphere soil (FNCA Biofertilizer Project Group, 2006).

The phospho-microorganism mainly bacteria and fungi make insoluble phosphorus available to the plants (NIIR Board, 2004). Several soil bacteria and a few species of fungi posses the ability to bring insoluble phosphate in soil into soluble forms by secreting organic acids (NIIR Board, 2004). These acids lower the soil pH and bring about the dissolution of bound forms of phosphate.

While *Rhizobium*, Blue Green Algae (BGA) and *Azolla* are crop specific, bio-inoculants like *Azotobacter, Azospirillum,* Phosphorus Solubilizing Bacteria (PSB), Vesicular Arbuscular *Mycorrhiza* (VAM) could be regarded as broad spectrum biofertilizers (NIIR Board, 2004). VAM is fungi that are found associated with majority of agriculture crops and enhanced accumulation of plant nutrients (NIIR Board, 2004). It has also been suggested that VAM stimulate plant growth by physiological effects or by reducing the severity of diseases caused by the soil pathogens (NIIR Board, 2004).

Examples of free living nitrogen fixing bacteria are obligate anaerobes (*Clostridium pasteurianum*), obligate aerobes (*Azotobacter*), facultative anaerobes, photosynthetic bacteria (*Rhodobacter*), cyanobacteria and some methanogens. The example of K-solubilizer is *Bacillus mucilaginous* while for P-solubilizer are *Bacillus megaterium, Bacillus circulans, Bacillus subtilis* and *Pseudomonas straita* (Wu *et al.*, 2004).

2.1.3 N-Fixer Bacteria

Nitrogen fixer or N-fixers organism are used in biofertilizer as a living fertilizer composed of microbial inoculants or groups of microorganisms which are able to fix atmospheric nitrogen. They are grouped into free-living bacteria (*Azotobacter* and *Azospirillium*) and the blue green algae and symbionts such as *Rhizobium*, and *Frankia* and *Azolla* (NIIR Board, 2004).

Rhizobium inoculation is well known agronomic practice to ensure adequate nitrogen of legumes instead of N-fertilizer (NIIR Board, 2004). In root nodules the O_2 level is regulated by special hemoglobin called leghemoglobin. This globin protein is encoded by plant genes but the heme cofactor is made by the symbiotic bacteria. This is only produced when the plant is infected with *Rhizobium*. The plant root cells convert sugar to organic acids which they supply to the bacteroids. In exchange, the plant will receive amino-acids rather than free ammonia.

Azolla biofertilizer is used for rice cultivation in different countries such as Vietnam, China, Thailand and Philippines. Field trial indicated that rice yields are increased by 0.5-2 tonnes/hectare due to *Azolla* application (NIIR Board, 2004). *Azobacter* and *Azospirillum* can fix atmospheric nitrogen in cereal crops without any symbiosis while blue-green algae have been found to be very effective on the rice and banana plantation (NIIR Board, 2004).

2.1.3.1 Growth Condition of N-Fixer

The different strains of the same species may have different sensitivity on pH medium. N-fixer bacteria can develop on media with pH range from 5.5 -9.0. Each individual species are differing in their sensitivity to an acid medium. Moreover, it is said that the optimum pH of N-fixer is lies between ranges 7.2 to 8.2 (Mishustin and Shilnikova, 1969). At both acidic and alkaline pH range, the growth of the bacteria will decrease (Dhanasekar, 2003). At this state, pH actually does affect the growth and nitrogen fixation.

Azotobacter is a typical mesophilic organism and most researchers regard 25-30ºC as the optimum temperature for *Azotobacter* (Mishustin and Shilnikova, 1969). At high temperature between 45-48ºC, vegetative *Azotobacter* cells cannot tolerate and will degenerated and dies (Mishustin and Shilnikova, 1969).

Growth condition can be experimented using shake flask. Generally, shake flasks experiment is used to optimize medium component while fermentor experiment is used to optimize operating condition.

2.1.4 Carriers Material for Biofertilizer

Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers (FNCA Biofertilizer Project Group, 2006). Sterilization of carrier material is essential to keep high number of inoculant bacteria on carrier for long storage period. Gamma-irradiation or autoclaving can be used as method for sterilization.

Various types of material can be used as carrier for seed or soil inoculation. According to Somasegaran and Hoben (1994), the properties of a good carrier material for seed inoculation are inexpensive and available in adequate amounts. It must non-toxic to inoculants bacterial strain and non-toxic to plant itself. Because it acts as carrier for seed inoculation, it should have good moisture absorption capacity and good adhesion to seeds. Last but not the least; carrier should have good pH buffering capacity, easy to process and sterilized by either autoclaving or gamma-radiation. Table 2.1 summarizes the carrier materials that can be used for inoculation and its characteristics.